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Monitoring Harmful Microalgae by Using a Molecular Biological Technique

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1. Introduction

In recent years, cultivation of fish and shellfish possesses an important portion for securing enough seafood all over the world. While, fisheries industry handling fish and shellfish derived from cultivation in addition to natural seafood are exposed to the danger of mass mortality of the reared and toxicities of bivalves, sometimes resulting in serious economic losses and physiological damages by seafood poisoning.

Certain microalgal species have been clearly demonstrated relationships with a mass mortality of fish and shellfish and certain symptoms of people which are caused by consumption of seafood contaminated with toxins. Occurrences of paralytic shellfish poisoning (PSP), neurotoxic shellfish poisoning (NSP), diarrheic shellfish poisoning (DSP), amnesic shellfish poisoning (ASP) and ciguatera fish poisoning (CFP) are caused through a food chain from toxin-producing microalgae to fish or shellfish (Hallegraeff 1995). Otherwise, some microalgal species cause a red tide, the name commonly used for the occurrence of harmful algal blooms (HABs) that result from local or regional accumulation of a unicellular phytoplankton species and exert a negative effect on the environment (Anderson 1994; Smayda 1997). Of the 5000 species of extant marine phytoplankton, approximately 300 algal species can form red tides, and the distribution of these HAB species is increasing globally. HABs therefore continue to receive attention in coastal regions all over the world (Hallegraeff 1993).

The canonical method monitoring HABs is that by observation of morphological features under a light microscope. This method requires labour, time, expert knowledge on morphologies of microalgae, and technical skills to observe the species-specific morphological features. In addition, morphology of microalgae is sometimes changed, depending on the environmental conditions or their growth phases (Imai 2000). Therefore, identification of HAB species with ambiguous morphology is quite difficult and sometimes subjective, and henceforth, problematic particularly in genera comprising both toxic and

non-toxic species which have similar morphology. The difficulty of monitoring HAB by light microscopy has indicated necessity of a more objective, rapid and accurate identification method for HAB species.

In the last decade, to address the above issue, molecular biological techniques have been developed for monitoring HAB species (Godhe et al. 2002; Sako et al. 2004; Hosoi-Tanabe and Sako 2005a). Many of such newly developed methods focus on genetic diversity of a certain gene that does not change in a short term depending on environmental conditions or the algal growth phase. This implies such molecular biological techniques can distinguish a HAB species from a morphologically similar but non-toxic species if the two species have gene sequences different from each other. Additionally, these assays appear to be time-saving, accurate, simple, and effective for the mass investigation of samples. So, polymerase chain reaction (PCR) assay, one of representative molecular biological techniques, is an indispensable tool in the fields regarding HAB-monitoring, since a PCR-based method allows us to identify or detect HAB cells more objectively even if they have morphology difficult to be defined their taxonomy under general microscopic methods (Adachi et al. 1994). Further, real-time PCR assay was later developed which allows us not only to detect and identify HAB species but also to quantify HAB cells (Bowers et al. 2000).

This is especially useful for resting cysts of certain HAB species. Some HAB species have a resting stage like a seed as one of their life cycles, and resting cysts of many HAB species are spheroid or ovate and with neither species-specific colour nor ornament (e.g., *Alexandrium*;).

In the chapter, we will introduce the principle of real-time PCR itself at first, and subsequently focus on several applications of the real-time PCR assay which have been developed to monitor dynamics of HAB species: e.g., neurotoxin-producing dinoflagellate *Alexandrium* species, red tide-forming dinoflagellates *Karenia mikimotoi* and *Cochlodinium polykrikoides*, red tide-forming raphidophytes *Chattonella* species and *Heterosigma akashiwo*, and bivalve-specific killer dinoflagellate *Heterocapsa circularisquama*.

Further, we introduce a method I and my coworkers have recently developed to process a lot of environmental seawater samples by using a filtration assay and the simplest protocol of DNA extraction (Shiraishi et al. 2009.). The simple method allows us to investigate many seawater samples for monitoring HAB species smoothly by using a real-time PCR assay with HAB species specific oligonucleotide primers and a probe.

2. Principle and application of real-time PCR assay for harmful algal blooms

In 1991, Holland et al. (1991) developed the new method called "*Taq* man real-time PCR assay", which is based on the 5′ to 3′-exonuclease activity of *Taq* polymerases, for monitoring the quantity of PCR product in real-time. Subsequently, the method was improved by Heid et al. (1996). The feature of the real-time PCR assay is requirement of a fluorogenic oligonucleotide probe in addition to reagents used in general PCR-based assay. The emission of 6-carboxy-tetramethyl-rhodamine (TAMRA) attached at the 3′-termini of probes as a quencher dye suppresses that of 6-carboxy-fluorescein (FAM) attached at the 5′-termini as a reporter dye due to the proximity between the emissions of two dyes. Describing the mechanisms of quantification briefly, the labeled probes hybridize with target DNA or PCR products and subsequently are deleted by the exonuclease activity in

each PCR-cycle, resulting in release of emission of the reporter dye. A fluorometer, which is generally equipped with a thermal cycler, detects the released emission of the reporter dye and quantifies the PCR products. Due to the utility, high-sensitivity, and accuracy of quantification, real-time PCR assay has been applied to development of a method for monitoring several HABs.

The first application of *Taq* man real-time PCR assay to HAB species was performed by Bowers et al. (2000) to quantitatively detect the toxic dinoflagellate *Pfiesteria piscicida* and its close relative *Pfiesteria shumwayae*. Bowers et al. (2000) designed primers-*Taq* man probe sets specifically hybridizes 18S rRNA gene of either *P. piscicida* or *P. shumwayae*. The real-time PCR assay using the primers-probe sets demonstrated high specificity even for single cells. Similar trials were carried out for the toxic dinoflagellate *Alexandrium* species (Galluzzi et al., 2004; Hosoi-Tanabe and Sako, 2005; Dyhrman et al. 2006), *Karenia brevis* (Gray et al. 2003), *Pfiesteria* spp. (Zhang and Lin, 2005), the naked harmful dinoflagellate *Cochlodinium polykrikoides*, *Karenia mikimotoi* (Kamikawa et al., 2006), and harmful raphidophytes (Handy et al., 2005; Bowers et al. 2006; Kamikawa et al., 2006).

Especially, *Taq* man real-time PCR assay was applied to resting cysts of *Alexandrium* species in marine sediments (Kamikawa et al. 2005, 2007; Erdner et al. 2011). The cyst densities calculated by the real-time PCR assay for *Alexandrium* cysts were almost identical to those by the canonical method to monitoring the cysts called primulin-staining (Yamaguchi et al. 1995, ; Kamikawa et al. 2007). However, it is notable that the cyst density calculated by the real-time PCR assay tends to be lower than that by the primulin method when sediment samples collected from 1-3cm depth were used (Erdner et al. 2011). This difference between the real-time PCR assay and the primulin method suggests that the real-time PCR assay may be influenced by cyst condition and viability (Erdner et al. 2011). Otherwise, there are unknown species that produce resting cysts with the similar morphology and that are stained with primulin as well.

3. The noxious dinoflagellate *Heterocapsa circularisquama*

The dinoflagellate *Heterocapsa circularisquama* is one of the most noxious phytoplankton in Japanese coastal areas and causes mass mortalities of both natural and cultured bivalves such as oyster, manila clam and pearl oyster in Japan (Nagai et al., 1996, 2000; Matsuyama, 1999). Blooms of this species have had significant negative impacts on the shellfish aquaculture especially in western coastal area of Japan (Matsuyama et al., 1997; Tamai, 1999). *H. circularisquama* was discovered for the first time in Uranouchi Inlet, Kochi Prefecture, Japan in 1988, and since that time, bloom occurrences have expanded throughout the western area of Japan (Matsuyama et al., 2001; Imai et al., 2006).

Monitoring the population dynamics of this species is essential for forecast of the red tide occurrences, and hence, for the mitigation of the damages, following to early countermeasures. Generalized seasonal occurrence of this species in summer and autumn could be determined using conventional optical microscopy (Matsuyama et al., 1996; Nakanishi et al., 1999; Shiraishi et al., 2007). However, precise identification and enumeration are difficult because this species is rather smaller than other red tide species (<30 μm), and there are numerous co-occurring dinoflagellates with similar morphology, implying that it is difficult to distinguish them from *H. circularisquama* (Horiguchi, 1995;

Iwataki et al., 2004; see also Fig. 1). Moreover, definitive identification of this species is based on morphology of body scales that can only be visualized using transmission electron microscopy (Horiguchi, 1995).

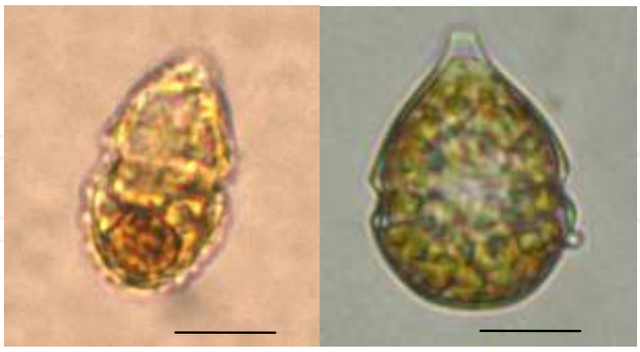

Fig. 1. Morphology of *Heterocapsa circularisquama* (left) and *Scrippsiella* sp. (right). Bar: 10μm.

Shiraishi et al. (2007) reported that it was possible to monitor *H. circularisquama* using an indirect fluorescent antibody technique (IFAT). This IFAT method allowed accurate detection of the cells even at low densities (lower limit, ca. 1 cell L^{-1}). Field studies using this method allowed the population dynamics of this species to be determined throughout a year in Uranouchi Inlet, Kochi Prefecture, Japan (Shiraishi et al. 2008.), and during early spring and later winter in Ago Bay, Mie Prefecture, Japan. Though the method demonstrated high specificity, individual *H. circularisquama* cells at lower density become difficult to be detected by epifluorescent microscopic observation in samples dominated by similar sized phytoplankton. This means additional treatments and significantly longer times were required to detect *H. circularisquama* cells in such samples. Consequently, there is still an urgent need to develop a simpler, quantitative method for monitoring *H. circularisquama*.

Kamikawa et al. (2006) previously reported a real-time PCR identification method of *H. circularisquama.* Though it could be used in the field, the assay as described requires a long and complex DNA extraction processes. Additionally, since target cells in cultures and seawater samples were collected by centrifugation, which imply that cells might be lost during the process and that only small sample volumes (50 mL at most) could be readily processed at a time. The conventional method using filtration was not feasible for concentrating *H. circularisquama* cells because most cells were attached and trapped on the surface of certain filters (Shiraishi et al., 2007). Thus, it was important to develop a simple technique for cell collection and DNA extraction to apply the *H. circularisquama*-specific real-time PCR assay to the field monitoring.

In our previous study, we developed a simple and quantitative monitoring method of *H. circularisquama* using a real-time PCR assay (Shiraishi et al. 2009.). The DNA extraction was performed within a relatively short time by gently filtering the cells down on a filter and then simply boiling the filter in a buffer. The population dynamics of *H. circularisquama* in an inlet revealed by the real-time PCR assay and by the IFAT assay were well consistent with each other. Because the method was only simply described in the original paper by the limitation of printing, we introduce the protocol of the simple real-time PCR assay (Shiraishi

et al. 2009.) in detail in the following sections. This protocol will be helpful for the studies on many other dinoflagellate species by real-time PCR assay.

4. Materials and methods

4.1 Organisms and culture conditions

The algal strains of *H. circularisquama*, *K. mikimotoi* and *Skeletonema* sp. were obtained from the National Research Institute of Fisheries and Environment of Inland Sea, Fisheries Research Agency. Strains of *Heterocapsa triquetra* and *Heterosigma akashiwo* were isolated by G. Nishitani from Maizuru Bay, Kyoto Prefecture, Japan in 1998 and by I. Imai from Hiroshima Bay, Hiroshima Prefecture, Japan in 1989, respectively. These strains were cultured at a temperature of 20 ^{o}C on a 14-h light: 10-h dark photo-cycle under an illumination at 180 μmol photons m^{-2} s^{-1} in modified SWM-3 medium (Chen et al., 1969; Imai et al., 1996).

4.2 Cell collection and DNA extraction

The most effective method of extracting *H. circularisquama* DNA from cell pellets was evaluated using six different protocols, the simplest of which was a TE boiling method modified from the procedure of Kamikawa et al. (2006). For the basic boiling procedure, either one cell or 100 cells of cultured *H. circularisquama* cells were collected on the Nuclepore polycarbonate membrane filters (pore size 3.0 μm) (Whatman, Maidstone, UK) by filtration, respectively. Extractions at both cell concentrations were done in triplicate to assess assay variability. The filter was placed in a 1.5-mL microtube without folding, and then 750 μL of TE buffer (10 mM Tris-HCl: pH 8.0, 1 mM EDTA: pH 8.0) was added. After the boiling for 10 min in the TE buffer, the filter was immediately removed. The extracted DNA sample was stored at -60 ^{o}C until the real-time PCR assay was performed. DNA extraction efficiency using the TE (Tris-HCl/EDTA) boiling method was compared with that by the modified CTAB (Cetyltrimethylammonium Bromide) method (Zhou et al., 1999; Kamikawa et al., 2005) and the proteinase K method (Kamikawa et al., 2005), both of which are commonly used. The same protocol as described above for the TE extractions was followed.

To choose the most suitable filter for the DNA extraction of *H. circularisquama*, the cells were collected on 6 different filters and the DNA extraction was performed on each filter by the TE boiling method which was found to be the efficient method from the study described above. Specifically, one cell and 100 cells of cultured *H. circularisquama* cells were collected by filtration onto membrane filters composed of either polycarbonate membranes (Nuclepore, mesh size 3.0 μm), glass-fibers (GF/C, pore size 1.2 μm) (Whatman, Maidstone, UK), cellulose mixed esters (pore size 3.0 μm) (Millipore, Tokyo, Japan), cellulose acetate (pore size 3.0 μm) (ADVANTEC, Tokyo, Japan), polytetrafluoroethylene (PTFE) (pore size 3.0 μm) (ADVANTEC, Tokyo, Japan), or hydrophilic polyvinylidene difluoride (PVDF) (pore size 5.0 μm) (Millipore, Tokyo, Japan), respectively. The DNA sample was stored at -60 ^{o}C until the real-time PCR assay was performed.

Based on the results of the extraction efficiency tests on the various tests, a standard curves consisting of eight-fold serial dilutions (10^{4} to 1 cells) of cultured cells were prepared. Each

number of the cells was collected on the Nuclepore filter (pore size 3.0 μm) (Whatman, Maidstone, UK) by filtration. The DNA extraction was performed by the TE boiling method, and the real-time PCR assay was carried out in triplicate. The standard curve was constructed based on the correlation between the threshold cycle (Ct value) and the number of cells.

A major concern when designing a real-time PCR assay for HABs is whether other co-occurring microalgae adversely affect the amplification efficiency either by introducing inhibitors or due to cross-reactivity problems. This possibility was explored in an examination where *H. circularisquama* cells (10^4 to 1 cells) were filtered on the Nuclepore filters (pore size 3.0 μm) at 20 cm Hg with 10^5 cells each of *H. triquetra*, *H. akashiwo*, *K. mikimotoi* and *Skeletonema* sp. which are frequently co-dominated in western coastal areas of Japan. A previous study also showed that the primers and probe used in this study are species-specific and do not react DNA from *H. triquetra*, *H. akashiwo* or *K. mikimotoi* (Kamikawa et al., 2006). The DNA extraction was performed by the TE boiling method, and the real-time PCR assay was carried out as follows in triplicate. Obtained Ct values at each number of cells were compared with those of the control experiment where only *H. circularisquama* was used.

4.3 Real-time PCR

The primer set and probe used in this study were based on unique species-specific DNA sites identified by aligning the D1/D2 LSU rDNA sequence of *H. circularisquama* (DDBJ/EMBL/GenBank accession number AB049709) with the correponding dinoflagellate sequences in GenBank. Primers specific to *H. circularisquama* were HcirF (5′-GTTTGCCTATGGGTGAGC-3′) and HcirR (5′-CATTGTGTCAGGGAGGAG-3′) and the probe was HcirTaqMan (5′-FAM-CACCACAAGGTCATGAGGACACA-TAMRA-3′) that was labeled at the 5′-end with FAM (carboxyfluorescein) and the 3′-end with TAMRA (carboxytetramethylrhodamine) (Kamikawa et al., 2006).

Thermal cycling was performed with a Rotor-Gene 3000 (Corbett Research, Mortlake, Australia) in 200-μL PCR tubes of commodity type. PCR was carried out in 25-μL volumes comprising 1×PCR EX Taq buffer (containing 20mM Mg2+), 200 μM dATP, 200 μM dTTP, 200 μM dGTP, 200 μM dCTP, 0.3 μM forward and reverse primers, 0.4 μM fluorogenic probe, and 1.25 U of Taq DNA polymerase (Takara EX TaqTM, TaKaRa Bio Inc., Shiga, Japan). The PCR conditions were as follows according to Kamikawa et al. (2006): one heating cycle at 95 ^{o}C for 2 min, followed by 45 cycles at 95 ^{o}C for 10 sec and 54 ^{o}C for 30 sec. The Ct value was calculated as a cycle number that an amplification curve reached at the most suitable threshold value.

5. Results and discussion

5.1 Development of a DNA extraction method

In order to examine the most efficient method for DNA extraction, three kinds of DNA extraction methods were subjected to *H. circularisquama* cells (100 cells and 1 cell) trapped on the filter. Figure 2 shows obtained Ct values for one and 100 cells by real-time PCR assay

with each DNA extraction method. For 100 cells, the DNA extracted with the TE boiling method was as efficient as with the CTAB method and the proteinase K method (t-test, df = 4, $p > 0.05$). For 1 cell, the DNA extraction efficiency with the TE boiling method was higher than that with the modified CTAB method (t-test, df = 4, $p < 0.05$) and similar to that of the proteinase K method (t-test, df = 4, $p > 0.05$). Thus, we can consider that the three methods are similarly efficient for *H. circularisquama* cells with high density. Given the importance of detection the HAB species at low density, the TE boiling method appeared to be the most useful technique for monitoring *H. circularisquama* by real-time PCR assay. In addition to its higher detection efficiency, the TE boiling method is more suitable in simplicity, ease of execution, lower cost, and shorter execution time than the other two methods.

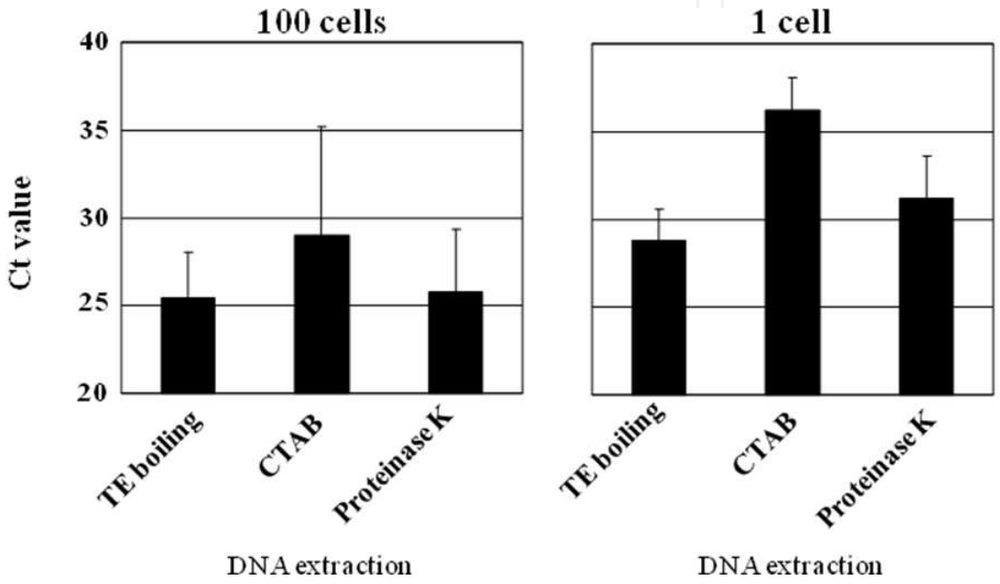

Fig. 2. Comparison of Ct (Threshold cycle) values obtained with three DNA extraction methods. TE boiling method, CTAB method and proteinase K and SDS method were subjected to 100 cells (left) and one cell (right) of *H. circularisquama* on Nucleporepolycarbonate membrane filters. Ct values were obtained by using the real-time PCR assay in triplicate. The bars show the standard deviations.

In order to select the filter which would yield the highest and most consistent recovery of DNA, samples containing either 1 or 100 cells of *H. circularisquama* cells were filtered onto six different types of filter. DNA was then extracted using the TE boiling method and subjected to real-time PCR-amplification. In the case of 100 cells, the real-time PCR assay successfully amplified *H. circularisquama* DNA from all the filters with the exception of the polytetra fluoroethylene (PTFE) membrane filter (Fig. 3). In contrast, for the 1 cell samples, the qPCR assay failed to reliably amplify the DNA from all the filters with the exception of the Nuclepore polycarbonate membrane filter, which could be detected in triplicate. Only one of three DNA samples extracted from either the cellulose acetate or hydrophilic polyvinylidine difluoride (PVDF) membrane filters were detected. Therefore, we concluded that the best filter for extraction and detection of *H. circularisquama* was Nuclepore polycarbonate membrane filter.

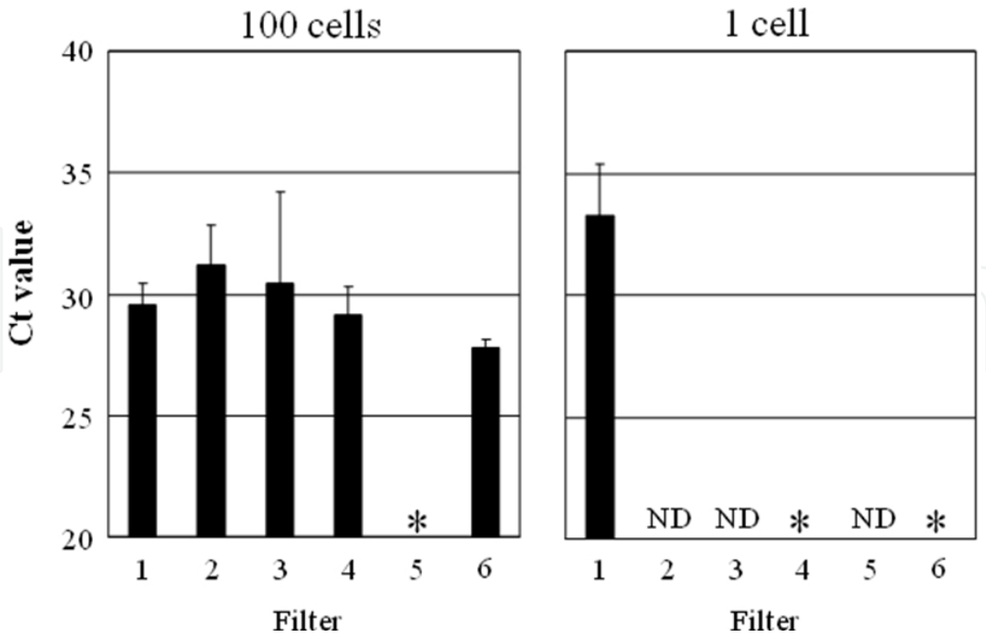

Fig. 3. Ct values obtained with TE boiling DNA extraction method for 100 cells (left) and 1 cell (right) collected on six different filters using the real-time PCR assay. 1, Polycarbonate filter; 2, Glass fiber filter; 3, Cellulose mixed ester filter; 4, Cellulose acetate membrane filter; 5, PTFE membrane filter; 6, Hydrophilic PVDF filter. Asterisks (*) indicate that Ct value could be obtained from only one of three filters. ND means that Ct value could not be obtained from all three filters. The bars show the standard deviations.

In a previous study (Kamikawa et al. 2006), *H. circularisquama* cells were concentrated by natural gravity filtration. However, it takes whole day for the concentration by natural filtration. It is not feasible for the routine works for monitoring natural populations. In addition, concentrating cells by filtration is not feasible for *H. circularisquama*, because most cells are attached and trapped on to the surface of any filters examined (Shiraishi et al., 2007).

Otherwise, more amount of seawater for monitoring is more suitable for detecting cell during period of low cell density, indicating that concentration of cells from seawater is important for accurate and sensitive detection.

The experiments above demonstrates that cultured cells of *H. circularisquama* can be quantitatively recovered and amplified from a single cell gently filtered onto Nucleopore filters (pore size 3.0 μm) and extracted using the boiling TE method (Fig. 4).

5.2 Validity of the real-time PCR assay

Serial dilutions of vegetative cells (1-10^4 cells) of *H. circularisquama* on Nuclepore filters were prepared, and then, the DNA was extracted by the TE boiling method. The real-time PCR assay was performed with the DNA samples in triplicates. The standard curve was constructed based on the mean Ct values and the number of *H. circularisquama* cells (Fig. 4). The obtained relationship between Ct values and the number of cells in logarithmic scale was linear, and the correlation coefficient was significantly high (r^2 = 0.997), indicating that the simple real-time PCR protocol can quantitatively detect *H. circularisquama* even from one cell.

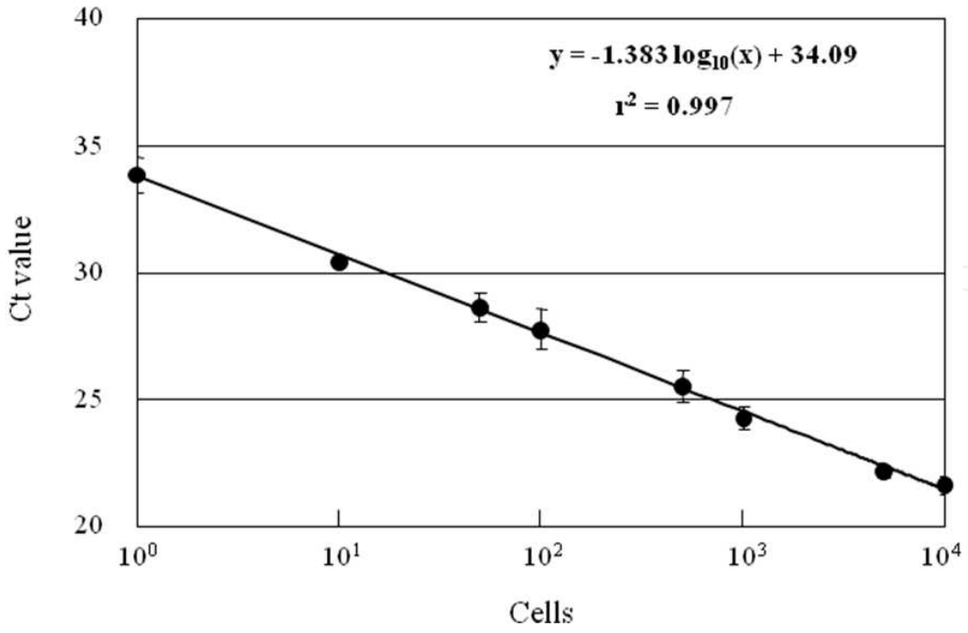

Fig. 4. Real-time PCR assay using eight-fold serial dilutions (10^4 to 1 cell). The result for each cell number was represented by each symbol shown in the figure.

In order to examine the effects of the existence of other microalgae on the DNA extraction or subsequent PCR-based quantification, *H. circularisquama* cells (10^4 to 1 cells) were collected together with 10^5 cells of *H. triquetra*, *H. akashiwo*, *K. mikimotoi* and *Skeletonema* sp. on the Nuclepore membrane filters by filtration. The DNA extraction and the real-time PCR assay were carried out as described above. The standard curve was constructed based on the mean Ct values and the number of cells (Fig. 5a). At each number of *H. circularisquama* examined, there was no significant difference between the Ct value obtained from *H. circularisquama* cells in spite of presence or absence of the other microalgae (t-test, df = 4, $p > 0.05$, Fig. 5b). The correlation between Ct values and the number of cells in logarithmic scale was linear, and the correlation coefficient was extremely significant ($r^2 = 0.991$, Fig. 5a). It was confirmed that the DNA extraction and subsequent PCR-based quantification of *H. circularisquama* cells were not inhibited even when other microalgae such as *H. triquetra*, *H. akashiwo*, *K. mikimotoi* and *Skeletonema* sp. coexist with *H. circularisquama*.

The constructed standard curve showed linearity (Fig. 4), and the protocol including concentration of cells, DNA extraction, and the real-time PCR was not inhibited by the existence of other microalgae even at 10^5 cells of *H. triquetra*, *H. akashiwo*, *K. mikimotoi* and *Skeletonema* sp. (Fig. 5b). It was clearly demonstrated that the presence of closely related species (e.g., *H. triquetra*) and/or many other common red tide species did not affect the efficiency of DNA extraction and subsequent PCR-based quantification of *H. circularisquama* cells.

When there are *H. circularisquama* cells in addition to much higher abundance of similar sized phytoplankton in the field, the detection of *H. circularisquama* cells by the canonical IFAT method with epifluorescent signal is obscured by the presence of numerous other microalgal cells. Similarly, an underestimation of cell abundance can be occurred when a large number of particles such as detritus not only inhibit epifluorescence microscopy

observation but also blocks the antibody reaction trapped within the detritus, indicating that the IFAT method is difficult to be applied to sediments and detritus-rich samples. The real-time PCR assay described in this study appears to be more feasible and practical for environmental samples than the IFAT method.

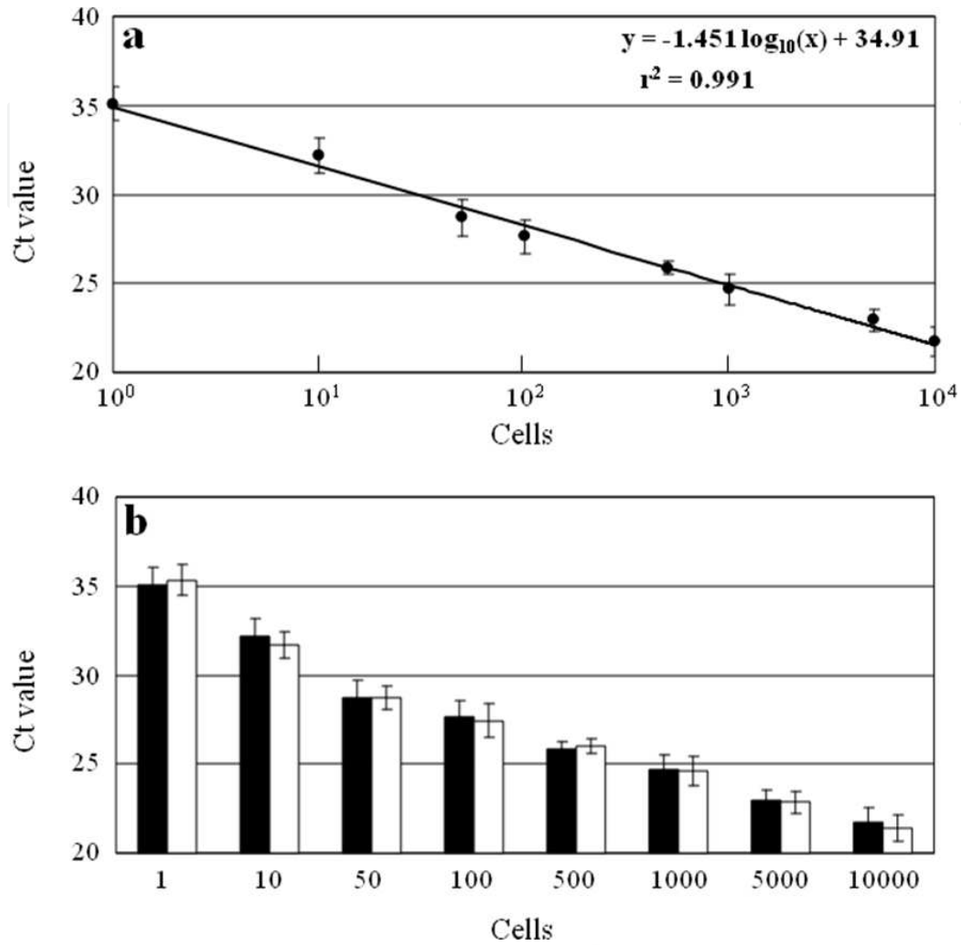

Fig. 5. Relationship between Ct values and the log number of cells. **a**. Standard curve for *H. circularisquama* cells constructed from DNA that was extracted from *H. circularisquama* cells plus several microalgae. **b**. Comparison of detection and quantification efficiency between DNA that was extracted from *H. circularisquama* cells (closed bars) and from *H. circularisquama* cells plus several microalgae (open bars). The bars show standard deviations.

6. Application to environmental samples

The procedure described above was applied to environmental samples in order to monitor successively *H. circularisquama* cells in addition to IFAT assay (Shiraishi et al. 2009.). The cell densities obtained by the real-time PCR assay were almost identical to the results obtained by the IFAT method. Hence, it was clearly demonstrated that *H. circularisquama* could be quantified by this simple real-time PCR assay as sensitively and precisely as the IFAT method in the field. It is notable that the detection limit of the real-time PCR assay was 1 cell/L: The most sensitive level currently with real-time PCR assay (Shiraishi et al. 2009.). It

should be also mentioned that the real-time PCR assay sometimes reacted to some environmental samples which the IFAT assay did not (Shiraishi et al. 2009.). This incongruence can be explained by that the real-time PCR assay is more sensitive than the IFAT assay. Otherwise, the real-time PCR assay might react to cell-free DNA derived from broken, dead cells of *H. circularisquama*. Since we have no idea which is true, it is better to use both methods for monitoring *H. circularisquama* cells in order to grasp precise dynamics of the HAB species, escaping both underestimation and overestimation.

In this chapter, it has been demonstrated that the real-time PCR assay can be applied to monitoring various HABs in field waters. If other HABs can be quantified by the same manner to present method with slight modification, those microalgae would be easily monitored with the similar procedures of the DNA extraction at the same time. The conventional methods for monitoring HABs with optical microscopy might be replaced by the simple real-time PCR assay in the near future, when the costs of machines and reagents are lowered to become reasonable.

In addition to seawater samples, real-time PCR assay has been applied for the detection of the cysts of the toxic *Alexandrium* species from marine sediments (Kamikawa et al., 2005, 2007, Erdner et al. 2011). Furthermore, the PCR method was also used for the detection of the cells of *Alexandrium* species in the tissue of mussels (Galluzzi et al., 2005) in order to investigate the possibility that the HAB cells are propagated to other areas by transport of bivalves. When *H. circularisquama* forms temporary cysts in water columns, those temporary cysts possibly sink down and survive some periods at the surface of the sea bottom. There are some reports that *H. circularisquama* could proliferate in water columns of a new area after the transportation of bivalves which accompany temporary cysts (Honjo et al., 1998; Honjo and Imada, 1999; Imada et al., 2001). Given the possibility of the temporary cysts as a seed-population, the detection of *H. circularisquama* cells is an urgent need from sediments, and tissues and fecal pellets of bivalves.

7. Acknowledgment

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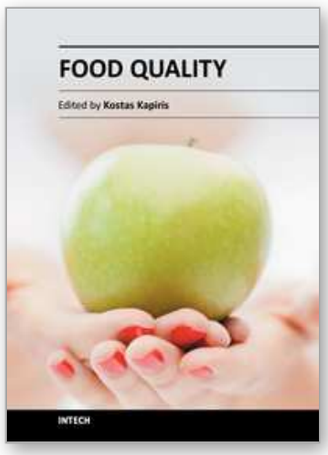

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The book discusses the novel scientific approaches for the improvement of the food quality and offers food scientists valuable assistance for the future. The detailed methodologies and their practical applications could serve as a fundamental reference work for the industry and a requisite guide for the research worker, food scientist and food analyst. It will serve as a valuable tool for the analysts improving their knowledge with new scientific data for quality evaluation. Two case study chapters provide data on the improvement of food quality in marine and land organisms in the natural environment.

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